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$p50^{Cdc37}$ functions in the establishment of protein kinase signaling pathways by functioning in complex with molecular chaperone Hsp90. Biochemical and genetic data indicate that Cdc37/Hsp90 complex plays a central role in the establishment of pathways that are directly implicated in cell cycle promotion and transformation. The proposed mode of function of Cdc37/Hsp90 complex is that Cdc37 targets intrinsically unstable kinases to the complex with Hsp90, and this transient interaction of newly synthesized kinases with the complex is necessary for their stabilization and/or folding and further activation. Multiple oncoproteins have been shown to require Hsp90/Cdc37 function for their stability. To test the hypothesis predicting that Cdc37 expression would be required for proliferative aspects of development and for cell proliferation induced by oncogenes, we created and analyzed a transgenic mouse model where Cdc37 expression is driven by MMTV promoter, directing the expression into breast epithelial cells, salivary and lacrimal gland, as well in some other organs. Our study show that Cdc37 is a first identified chaperone functioning as an oncogene, and is capable of cooperating with other oncogenes such as c-*myc* and *neu* for transformation.

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Role of Cdc37 in breast cancer

PI Lilia J. Stepanova

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INTRODUCTION

$p50^{Cdc37}$ functions in the establishment of protein kinase signaling pathways by functioning in complex with molecular chaperone Hsp90. Biochemical and genetic data indicate that Cdc37/Hsp90 complex plays a central role in the establishment of pathways that are directly implicated in cell cycle promotion and transformation. The proposed mode of function of Cdc37/Hsp90 complex is that Cdc37 targets intrinsically unstable kinases to the complex with Hsp90, and this transient interaction of newly synthesized kinases with the complex is necessary for their stabilization and/or folding and further activation. Multiple oncoproteins have been shown to require Hsp90/Cdc37 function for their stability. To test the hypothesis predicting that Cdc37 expression would be required for proliferative aspects of development and for cell proliferation induced by oncogenes, we created and analyzed a transgenic mouse model where Cdc37 expression is driven by MMTV promoter, directing the expression into breast epithelial cells, salivary and lacrimal gland, as well in some other organs. Our study show that Cdc37 is a first identified chaperone functioning as an oncogene, and is capable of cooperating with other oncogenes such as c-*myc* and *neu* for transformation.

RESULTS AND DISCUSSION

1. Cdc37 is an oncogene.

We created two lines of transgenic mice expressing Cdc37 under control of MMTV promoter and confirmed the insertion of the transgene by Southern analysis of tail DNA. The expression pattern of the Cdc37 was characterized by the combination of Northern blot and immunohistochemistry.

MMTV-*CDC37* lines and control littermates were maintained as breeding colonies and monitored for developmental and transformation phenotypes for up to two years. Transgenic animals appeared normal at birth, and their growth was indistinguishable from their non-transgenic littermates. Their reproduction, number of pups per litter, and their lactation in females were normal, although promiscuous male breast development was detected (see below).

Malignant transformation of the mammary gland or other organs was not observed during first year of life in *CDC37* transgenic animals. However, as MMTV-*CDC37* animals approached 18 months of age, a significant fraction of animal from both lines developed proliferative disorders, including mammary tumors and lymphomas. Histological analysis indicated that mammary tumors were adenocarinomas and adenosquamous cancinomas. Immunohistochemistry revealed high levels of Cdc37 protein expression in a large fraction of tumor cells, while only 5-10% of the surrounding unaffected ductal epithelial cells expressed *CDC37*. Lymphomas in transgenic females usually manifested themselves as an extreme weakness of the animals, and obvious enlargement of lymph nodes.

Control non-transgenic female littermates from the same age group displayed no evidence of proliferative disorders. Non-transgenic control animals were subjected to a detailed pathological analysis either in parallel with *CDC37* transgenic animals or at 23-24 months of age. While all of the transgenic females in one line, and most in another have developed tumors, males of both lines, as well as non-transgenics, did not develop any kind of malignancies up to 2 years of age.

2. Cdc37 cooperates with c-*myc* and *neu* oncogenes.

CDC37 is expressed in proliferative zones in adult tissues and is co-expressed with cyclin D1 in several tissues, but is absent in many differentiated cell types, including many epithelial cell types (Stepanova, L., Leng, X., Parker, S., Harper, J.W. Mammalian p50Cdc37 is a protein kinase-targeting subunit of Hsp90 that binds and stabilizes Cdk4. Genes and Development 1996; 10, 1491-1502). We hypothesized that *CDC37* expression might be required to support transformation by oncogenic pathways. In this case, we would predict that inappropriate *CDC37* expression might promote proliferative events dependent on oncogenic pathways.

To test this, we crossed MMTV-*CDC37* heterozygous females with MMTV-c-*myc* and MMTV-*neu* homozygous males. We evaluated the influence of the level of expression of the transgene on tumorigenesis by dividing single and double transgenic females into two groups, one which was kept virgin, and another which was kept in the presence of breeder males.

Cooperation of Cdc37 with c-*myc* was observed in the induction of mammary tumorigenesis in both virgin and breeding group of females, where in addition to acceleration of tumor incidence, we also observed a dramatic increase in the number of tumors/animal. In addition to accelerated kinetics of tumor incidence in virgin females, we observed altered tissue specificity of transformation in *CDC37*/c-*myc* double transgenic females. In double transgenic females, a prevalent tumor type was salivary adenocarcinoma. This tumor type has never been reported in c-*myc* expressing animals, although c-*myc* expression in the salivary gland was detected previously. Adenocarcinomas found in double transgenics appeared to be fast-growing, with many mitotic figures. Taken together, these data indicate that MMTV-*CDC37* can alter the rates and extent of transformation in both breeding and non-breeding MMTV-c-*myc* mice, and can also alter the specificity of transformation.

MMTV-c-*myc* expressing males are typically free of proliferative disorders. Therefore we were surprised to find evidence of both overt Leydig cell tumors and testicular hyperplasia in double transgenic males. Cdc37 is normally not detectable in the testis of normal mice but is readily apparent in Leydig cells in MMTV-*CDC37* mice. Leydig cell tumors were observed in MMTV-*CDC37*+c-*myc* males as early as 10 months of age. At an age of ~ 400 days, about 2/3 of all apparently unaffected males were sacrificed and their testes were subjected to detailed histological analysis. A significant fraction (75%) of double transgenic males displayed overt Leydig cell hyperplasia, a possible precursor to overt transformation. In contrast, only about 20% of MMTV-c-*myc* males displayed Leydig cell hyperplasia and the extent of overproliferation was modest.

Neu is a potent oncogene and a large fraction of MMTV-*neu* mice display proliferative disorders in the mammary gland within 10 months of birth. We monitored MMTV-*neu* and MMTV-*CDC37*/*neu* mice for transformation. The kinetics of tumor appearance was similar in MMTV-*neu* and MMTV-*CDC37*+*neu* females (T_{50}~350 days). All tumors were found to be mammary adenocarcinomas of either ductal or alveolar origin. Both single and double transgenic mice usually developed a single tumor mass with no evidence of metastasis. Histological analysis revealed that despite a similar gross appearance and incidence, MMTV-*neu* and MMTV-*CDC37*/*neu* tumors display dramatically different numbers of mitotic figures. While mitotic figures were rare in MMTV-*neu* tumors (1-2 per microscopic field), we observed ~8 mitotic figures per field in tumors from MMTV-*CDC37*+*neu* mice. These data indicate that CDC37 expression does not influence the frequency of transformation by *neu* but may enhance proliferative signals downstream of *neu*.

3. Biochemical characterization of Cdc37/Hsp90 targets in transformed cells.

To begin to address Specific Aim #3 and investigate how *CDC37* and c-*myc* collaborate in transformation, we examined the levels of several protein kinases as well as c-*myc* in mammary carcinomas from MMTV-*CDC37*/c-*myc* and MMTV-c-*myc* animals. As a control, we also examined the levels of proteins in MMTV-*ras* tumors. The level of Cdk4 and Erk1 kinases was increased in tumors expressing MMTV-*CDC37*, relative to that found with MMTV-c-*myc* alone. Unexpectedly, we found that c-*myc* levels were also increased in the presence of MMTV-*CDC37*, compared to animals expressing only MMTV-c-*myc*. The observed differences in protein levels cannot be explained by the increased number of dividing cells, since no significant difference was observed in the mitotic index of these tumors. Tumors derived from MMTV-*ras* and MMTV-c-*myc* mice contained a single Cdc37 species while higher levels of a closely spaced doublet of Cdc37 protein was found in extracts from MMTV-*CDC37*+c-*myc* tumors. Multiple forms of Cdc37 have been observed proviously (Stepanova, L., Leng, X., Parker, S., Harper, J.W. Mammalian p50Cdc37 is a protein kinase-targeting subunit of Hsp90 that binds and stabilizes Cdk4. Genes and Development (1996) 10, 1491-1502) and may reflect its phosphorylation (unpublished data, L.S. and J.W.H.). Recent studies suggested that activation of the *ras* pathway stabilizes c-*myc* (Sears et al., 1999. Ras enhances Myc protein stability. Mol. Cell 3, 169-79.). It is therefore possible that *CDC37*, through its interaction with kinases in the *ras* pathway, indirectly stabilizes c-*myc*. Further studies are required to determine whether increased levels of c-*myc* via *CDC37* expression are an important component of the collaborative effects seen in vivo.

Since we observed a striking cooperation between c-*myc* and *CDC37* in induction of mammary tumors, we analyzed *CDC37* targets in tumors derived from c-*myc* and *CDC37*/c-*myc* animals. Analysis of tumors has many advantages over analysis of the breast cancer cell lines, since cell lines are prone to acquiring additional somatic mutations complicating analysis. The potential mechanism of cooperation between *CDC37* and c-*myc* was proposed based on our biochemical analysis, and the data is prepared for publication (see below).

The task 11 and 13 of the grant proposal aimed at the development of dominant-negative mutant of *CDC37* for facilitation of the analysis of *CDC37* complex composition. The generation of dominant negative mutant of *CDC37* and detailed characterization of its effect of the cellular transformation was reported by others during our work on the project (Grammatikakis, et al. p50Cdc37 acting in concert with Hsp90 is required for Raf-1 function. Mol Cell Biol 1999 19: 1661-1672).

4. Inappropriate mammary duct development in male MMTV-*CDC37* mice.

Phenotypic analysis of mammary glands during development failed to identify significant differences between female MMTV-*CDC37* mice and their wild-type littermates, except for a 2-3 days delay in the rate of involution after lactation (data not shown). However, we did observe alterations in the development of male ductal

systems, as assessed by whole-mount analysis. The degree of breast duct development varies in different mouse strains, ranging from the presence of the initial ductal sprout in some of the fat pads to relatively well-developed branching ductal tree. In our strain background, male mice do not develop a significant mammary duct structure, although the fat pad is well-developed. In contrast, 60-70% of the adult MMTV-*CDC37* male mice have well-formed breast ducts with different degrees of elaboration by the age of 7 months. In the MMTV-*CDC37.2* line, which has lower levels of expression, 30-40% of male animals developed breast ducts in the inguinal fat pad by the age of 7 months. In control non-transgenic littermates of the similar mixed background, only 10% of adult males have non-branching initial sprout structure.

5. Generation of PB-Cdc37 mice and effects of Cdc37 in the prostate.

Cdc37 expression is absent in normal human prostate epithelium but is highly induced after transformation. Interestingly, Cdc37 induction is apparent in some pre-malignant lesions, probably reflecting the fact that Cdc37 induction is a early event in transformation. To test the effect of Cdc37 expression in normal prostate, Cdc37 ORF was placed under control of PB (probasin) promoter which directs expression selectively to the prostate. SV40 splice site and polyadenylation sequences were added to ensure the proper post-translational processing of the mRNA. Two lines of transgenic animals were generated, and inheritability of the insert was ensured. Prostates of transgenic and control non-transgenic animals were fixed, embedded in paraffin, sectioned, stained with H&E and analyzed under the microscope. Close to 80% of the transgenic males in both lines developed proliferative disorders in the prostate epithelium, including high-grade hyperplasias and cribriform structures, further confirming cell-cycle promoting capabilities of *CDC37*.

6. Analysis of MMTV-*CDC37*/cyclin D1 double transgenic animals.

To continue analyzing the effects of simultaneous expression of Cdc37 with other oncogenes, we generated mice expressing both Cdc37 and cyclin D1. Each of the oncogenes cause tumor development starting at the age of 16-20 months. Double transgenic females in our experiment are now 12 months old and started to show signs of transformation. We will further observe these animals for the signs of cooperation.

KEY RESEARCH ACCOMPLISHMENTS

* Discovery of the oncogenic properties of *CDC37*, first oncogene of chaperone class

* *CDC37* is able to cooperate with c-*myc* in transformation of multiple tissues

* *CDC37* is able to cooperate with *neu* in increasing the mitotic index of the mammary tumors

* MMTV-*CDC37* animals have developmental defects, which is consistent with its positive role in promoting cell division

* PB-*CDC37* males develop neoplastic changes in prostate

REPORTABLE OUTCOMES

The results 1-4 are submitted for the publication to **The EMBO Journal** (Lilia Stepanova, Milton Finegold, Franco DeMayo, and J. Wade Harper, 1999. The Oncoprotein Kinase Chaperone $p50^{Cdc37}$ Functions as an Oncogene and Collaborates with c-myc in Transformation of Multiple Tissues; see attached manuscript).

The result 5 is in the process of being prepared for publication.

The results of the study have been presented on national symposia:

L. Stepanova, MJ. Finegold, and JW. Harper (1998). Altered Proliferation and Tumorigenesis in MMTV-Cdc37 Transgenic Mice and Potential Collaboration with Myc in Induction of Salivary Gland Tumors. 51st Annual Symposium on Fundamental Cancer Research: Molecular Targets for Cancer Therapy and Prevention, Houston, TX

L. Stepanova, MJ. Finegold, and JW. Harper (1999). Oncoprotein Kinase Chaperone Cdc37 is a New Oncogene Able to Cooperate with Other Oncogenes. 28th National Student Research Forum, Galveston, TX

L. Stepanova, MJ. Finegold, and JW. Harper (1999). Cdc37 is a first protein chaperone with properties of an oncogene. 1999 Biochemistry and Molecular Biology Meeting/ ASBMB Satellite Meeting, San Francisco, CA.

APPENDICES

1. Lilia Stepanova, Milton Finegold, Franco DeMayo, and J. Wade Harper (1999). The Oncoprotein Kinase Chaperone $p50^{Cdc37}$ Functions as an Oncogene and Collaborates with c-myc in Transformation of Multiple Tissues (submitted).

The Oncoprotein Kinase Chaperone $p50^{Cdc37}$ Functions as an Oncogene and Collaborates with c-myc in Transformation of Multiple Tissues

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Abstract

CDC37 encodes a 50 kDa protein that functions to target intrinsically unstable oncoprotein kinases including Cdk4, Raf-1, and v-src to the molecular chaperone Hsp90, an interaction that is important for establishment of signaling pathways. *CDC37* is an essential gene in budding yeast and is co-expressed with cyclin D1 in proliferative zones during mouse development, consistent with a positive role in cell proliferation. *CDC37* expression may be required not only to support proliferation in cells that are developmentally programmed to proliferate but also in those that are inappropriately induced to initiate proliferation by oncogenes. Here we report that MMTV-*CDC37* transgenic mice develop mammary gland tumors at a rate comparable to that observed previously in MMTV-cyclin D1 mice. Moreover, *CDC37* was found to collaborate with MMTV-c-*myc* in transformation of multiple tissues, including mammary and salivary glands in females, and testis in males. These data indicate that *CDC37* can function as an oncogene in mice and suggest that the establishment of protein kinase pathways mediated by Cdc37/Hsp90 can be a rate-limiting event in epithelial cell transformation.

Introduction

Extracellular signals act to coordinate proliferation during the first gap (G1) phase of the cell division cycle. These signals typically act through receptor tyrosine kinases to activate protein kinase signaling pathways that direct the expression of genes required for proliferation. Recent studies have implicated components of the *ras* pathway in regulating the expression of D-type cyclins, a central component of mitogen dependent cell cycle entry (Aktas et al., 1997; Peeper et al., 1997). Ras activation leads to engagement of the Raf/MEK/MAPK pathway (Robbins et al., 1992; Thomas et al., 1992; Wood et al., 1992; Warne et al., 1993; Zhang et al., 1993), and each of these components is necessary and sufficient to induce cyclin D expression (Albanese et al., 1995; Lavoie et al., 1996; Winston et al., 1996; Aktas et al., 1997; Kerkhoff and Rapp, 1997; Pepper et al., 1997). D-type cyclins are essential activator subunits of Cdk4 and Cdk6, and holoenzyme complexes of these kinases have been implicated in cell cycle entry through multiple mechanisms. Cyclin D/Cdk4 complexes directly phosphorylate Rb and initiate inactivation of its growth suppressor function (Ewen et al., 1993; Kato et al., 1993; Matsushime et al., 1994; Meyerson and Harlow, 1994; Connell-Crowley et al., 1997). In addition, cyclin D/Cdk4 complexes may contribute to activation of cyclin E/Cdk2 by titrating the Cdk inhibitor $p27^{KIP1}$ from Cdk2 complexes (Kato et al., 1994; Sherr and Roberts, 1995; Reynisdottir et al., 1995; Reynisdottir and Massague, 1997; Cheng et al., 1998; McConnell et al., 1999). Consistent with the central role of cyclin D in ras dependent proliferation is the finding that Cdk4 inhibitors of the p16 class can inhibit ras-mediated proliferation in an Rb-dependent manner (Lukas et al., 1995; Serrano et al., 1995; Mittnacht et al., 1997; Peeper at al., 1997).

The assembly of cyclin D/Cdk4 complexes is complex and appears to involve multiple steps, including a mitogen dependent step (Matsushime et al., 1994; Meyerson and Harlow, 1994; LaBaer et al., 1997; Cheng et al., 1998, 1999). Previously, we cloned a mammalian homolog of the budding yeast *CDC37* gene and demonstrated that $p50^{Cdc37}$ binds to Cdk4 and Cdk6, but not Cdc2 and Cdk2 (Stepanova et al., 1996). In budding yeast, *CDC37* is an essential gene and is required for formation of Cdc28/Cln complexes through an unknown mechanism (Gerber et al., 1995). We and others demonstrated that mammalian Cdc37 assembles with Cdk4 in high molecular weight complexes that also contain the molecular chaperone Hsp90 (Dai et al., 1996; Stepanova et al., 1996; Lamphere et al., 1997). We also demonstrated that the *CDC37* gene encodes the Hsp90-associated p50 protein (Stepanova et al., 1996), previously seen in complexes with v-src (Brugge, 1981, 1986; Whitelaw et al., 1991; Hutchison et al., 1992) and Raf (Stancato et al., 1993) but whose identity was unknown. Cdc37 associates with Hsp90 independent of protein kinases and appears to function at least in part as a protein kinase targeting subunit of Hsp90 (Stepanova et al., 1996). Genetic and biochemical data in several systems suggests that particular protein kinases are intrinsically unstable and their association with the Cdc37/Hsp90 chaperone is important for folding and/or activation of the targeted kinase (Cutforth and Rubin, 1994; Gerber et al., 1995; Stepanova et al., 1996; Grammatikakis et al., 1999; Munoz and Jimenez, 1999; Xu et al., 1999). Once Cdk4 is stabilized by the Cdc37 complex, it is released in a step that is not characterized, and can then assemble with either inhibitors such as p16 or with cyclin D. Assembly with cyclin D appears to involve the action of p21 and/or p27, possibly in addition to a mitogen-dependent step (LaBaer et al., 1997; Cheng et al., 1999; Parry et al., 1999).

Previously, we demonstrated that *CDC37* is expressed primarily in proliferative zones during embryonic development and in adult tissues, and its pattern of expression closely corresponded to that of cyclin D1 (Stepanova et al., 1996). Interestingly, *CDC37* is not expressed in several adult tissues including virgin mammary duct epithelial cells but, like cyclin D1, is induced during pregnancy, consistent with a positive role in proliferation (Stepanova et al., 1996). These data, coupled with the fact that *CDC37* is required for proliferation in budding yeast and *Drosophila,* suggest that *CDC37* expression may be required to support proliferation not only in those cells that are developmentally programmed to proliferate but also in those that are inappropriately induced to initiate proliferation by oncogenes. If this were the case, then *CDC37* would be predicted to collaborate with transforming oncogenes. To examine this question, we generated MMTV-*CDC37* transgenic mice, which allows expression of *CDC37* in the mammary gland and other differentiated tissues where it is normally not present. MMTV-*CDC37* transgenic mice were found to develop mammary gland tumors at a rate comparable to that observed in MMTV-cyclin D1 mice. Moreover, *CDC37* was found to collaborate with MMTV-c-*myc* in transformation of multiple tissues, including mammary and salivary glands in females, and testis in males. These data indicate that Cdc37 can function as an oncogene in mice and suggest that the establishment of protein kinase pathways mediated by Cdc37/Hsp90 can be a rate limiting event in epithelial cell transformation.

Results

Construction and *CDC37* expression in MMTV-*CDC37* transgenic mice.

To assess the possible role of *CDC37* in promoting neoplastic transformation, transgenic mice expressing *CDC37* under control of MMTV promoter (Figure 1A) were generated. Two transgenic founders were produced which transmitted the transgene to their progeny in a Mendelian fashion and both contain multiple copies of the transgene (Figure 1B). Lines of transgenic animals (MMTV-*CDC37*.1 and MMTV-*CDC37*.2) were established by mating each founder with outbred ICR mice. Expression of the *CDC37* transgene was observed in several tissues derived from adult MMTV-*CDC37*.1 and MMTV-*CDC37*.2 animals, including lacrimal, mammary, and salivary glands, uterus, and testis, by Northern analysis using both the *CDC37* cDNA and transgene specific regulatory sequences (5'+3') as probes. The levels of mRNA in the MMTV-*CDC37*.2 strain was ~50% of those in the MMTV-*CDC37*.1 line (Figure 1C and data not shown), consistent with the lower number of transgenes (Figure 1B). This pattern of expression is consistent with what has been observed previously with other MMTV-driven transgenes. The levels of transgene mRNA are significantly higher than the levels of endogenous *CDC37* message in specific tissues. However, the levels of Cdc37 protein in particular tissues derived from MMTV-*CDC37*.1 mice, including mammary epithelium, salivary gland, and testis, were comparable to the levels seen in proliferating mammary epithelium from normal mice during pregnancy or in fibroblasts maintained in tissue culture, as determined by immunofluorescence (see Figures 4 and 6 and data not shown).

CDC37 is an oncogene.

MMTV-*CDC37* lines and control littermates were maintained as breeding colonies and monitored for developmental and transformation phenotypes for up to two years. Transgenic animals appeared normal at birth, and their growth was indistinguishable from their non-transgenic littermates. Their reproduction, number of pups per litter, and their lactation in females were normal, although promiscuous male breast development was detected (see below).

Malignant transformation of the mammary gland or other organs was not observed during first year of life in *CDC37* transgenic animals. However, as MMTV-*CDC37* animals approached 18 months of age, a significant fraction of animal from both lines developed proliferative disorders, including mammary tumors and lymphomas (Table 1, Figure 2 and 3A). Histological analysis indicated that mammary tumors were adenocarinomas and adenosquamous cancinomas (Figure 2). By 22 months of age, all 10 MMTV-*CDC37*.1 females observed had developed tumors (Figure 3A). Mammary tumors in MMTV-*CDC37*.1 mice arose as singular persistent masses, which failed to show any detectable increase in size for up to two months after initial detection. Histopathological sections revealed very slow growing carcinomas with enlarged nuclei, and frequent keratin deposits indicating squamous differentiation (Figure 2A,B). The tumors arose adjacent to normal mammary epithelium and were clearly distinguishable from it. Consistent with the apparent slow growth rate, mitotic figures were rare in these tumors. Necrotic and apoptotic changes were minimal. Immunohistochemistry revealed high levels of Cdc37 protein expression in a large fraction of tumor cells (Figure 2A,c and d; and 2B,c and d), while only 5-10% of the surrounding unaffected ductal epithelial cells expressed Cdc37 (not shown). Heterogeneous MMTV transgene expression in the

mammary epithelium is frequently observed. Lymphomas in transgenic females usually manifested themselves as an extreme weakness of the animals, and obvious enlargement of lymph nodes. Two cases of lymphomas were discovered in animals that already had developed mammary adenosquamous carcinomas. All lymphomas exhibited very low mitotic activity, which could explain the slow progression of disease (Figure 2C).

Eleven animals of the MMTV-*CDC37*.2 line were autopsied at 17 months of age. Seven of these displayed evidence of proliferative disorders (Table 1). Tumors found included two cases of lymphoma, one mammary adenosquamous carcinoma, two liposarcomas, one case of fibrosarcoma, and a lung adenocarcinoma. As in the first line examined, all tumors displayed a low mitotic index with little evidence of apoptosis.

Control non-transgenic female littermates from the same age group displayed no evidence of proliferative disorders (Figure 3A). Non-transgenic control animals were subjected to a detailed pathological analysis either in parallel with *CDC37* transgenic animals or at 23-24 months of age. While all of the transgenic females in one line, and most in another have developed tumors, males of both lines, as well as non-transgenics, did not develop any kind of malignancies up to 2 years of age.

***CDC37* cooperates with c-*myc* in induction of mammary tumors in breeding females.**

CDC37 is expressed in proliferative zones in adult tissues and is co-expressed with cyclin D1 in several tissues, but is absent in many differentiated cell types, including many epithelial cell types (Stepanova et al., 1996). We hypothesized that *CDC37* expression might be required to support transformation by oncogenic pathways. In this case, we would predict that inappropriate *CDC37* expression might promote proliferative events dependent on oncogenic pathways.

To test this, we crossed MMTV-*CDC37* heterozygous females with MMTV-c-*myc* and MMTV-*neu* homozygous males. To control for differences in genetic backgrounds, we monitored heterozygous c-*myc* and *neu* littermates alongside the double transgenics. Previously it was shown that multiple rounds of pregnancy and lactation are able to promote expression of the c-myc transgene and accelerate tumorigenesis (Steward et al., 1984). We evaluated the influence of the level of expression of the transgene on tumorigenesis by dividing single and double transgenic females into two groups, one which was kept virgin, and another which was kept in the presence of breeder males.

Tumors were observed in breeding MMTV-c-*myc* females as early as 3 months of age and 50% of females had developed tumors by 250 days of age in this genetic background (Figure 3A). In contrast, breeding females carrying both c-*myc* and *CDC37* transgenes developed tumors with accelerated kinetics and 50% of females developed tumors by the age of 115 days (Figure 3A). All tumors developed by breeding females were mammary ductal and alveolar adenocarcinomas (Figure 3B,E,F). In addition to acceleration of tumor incidence, we also observed a dramatic increase in the number of tumors/animal (Figure 3C, and D). This included both an increase in the number of glands affected as well as the number of tumors/gland (Figure 3D). While MMTV-c-*myc* animals rarely had all of the glands affected, virtually all of the double transgenic animals were affected in every gland. While MMTV-c-*myc* females had on average 3 tumors per animal, MMTV-*CDC37*+c-*myc* approached 20 tumors per animal, on average (Figure 3D). In many cases, the tumor masses were so abundant, it prevented an exact determination of tumor foci. On sections of both MMTV-*CDC37*+c-*myc* and MMTV-c-

myc mammary glands all transitions from normal to transformed epithelium could be seen, including multiple areas of hyperplasia.

Altered tissue specificity of transformation in non-breeding MMTV-*CDC37*+c-*myc* females.

CDC37 is normally not expressed in virgin mammary epithelium but is induced during pregnancy. c-*Myc* has been shown to induce mammary transformation in virgin mice in some genetic backgrounds, although the extent of transformation is much lower than observed with multiple pregnancies. To examine whether *CDC37* can collaborate with c-*myc* in the absence of hormonal stimulation, we examined females maintained in the virgin state. Virgin females incurred palpable tumors later in life, around 16 months for females carrying both transgenes, and around 19 months for MMTV-c-*myc* animals (with the exception of one female developing a T-cell lymphoma which is not characteristic of virgin MMTV-c-*myc* mice) (Figure 3A). Also, in our strain background, MMTV-c-*myc* virgin females typically incurred B-cell lymphomas as opposed to mammary carcinomas (Figure 3B). The kinetics of tumor development was very slow, and only 25% of females developed tumors by the age 500 days. In contrast, the kinetics of tumor incidence in double transgenics was substantially accelerated (Figure 3A) and the spectrum of tumors was much wider (Figure 3B), including both T- and B-cell lymphomas, as well as mammary and salivary gland adenocarcinomas. In double transgenic females, a prevalent tumor type was salivary adenocarcinoma (Figure 3B). In normal salivary glands, Cdc37 protein is not detectable but is readily observed in the salivary epithelium of MMTV-*CDC37* mice and throughout tumors derived from double transgenics (Figure 4A-C). This tumor type has never been reported in c-myc expressing

animals, although c-*myc* expression in the salivary gland was detected previously (Stewart et al., 1984). Adenocarcinomas found in double transgenics appeared to be fast-growing, with many mitotic figures (Fig. 4C, a). Taken together, these data indicate that MMTV-*CDC37* can alter the rates and extent of transformation in both breeding and non-breeding MMTV-c-*myc* mice, and can also alter the specificity of transformation.

Testicular hyperplasia and transformation in MMTV-*CDC37*+c-*myc* males.

MMTV-c-*myc* expressing males are typically free of proliferative disorders (Steward et al., 1984). Therefore we were surprised to find evidence of both overt Leydig cell tumors and testicular hyperplasia in double transgenic males (Figure 5). Cdc37 is normally not detectable in the testis of normal mice but is readily apparent in Leydig cells in MMTV-*CDC37* mice (Figure 5D). Leydig cell tumors were observed in MMTV-*CDC37*+c-*myc* males as early as 10 months of age (Figure 5A, G). One of the four animals had two distinct Leydig cell tumors one in each testis. At an age of ~ 400 days, about 2/3 of all apparently unaffected males were sacrificed and their testes were subjected to detailed histological analysis. A significant fraction (75%) of double transgenic males displayed overt Leydig cell hyperplasia (Figure 5F), a possible precursor to overt transformation. In contrast, only about 20% of MMTV-c-*myc* males displayed Leydig cell hyperplasia and the extent of overproliferation was modest (Figure 5B, C, E).

Biochemical analysis of tumors derived from breeding MMTV-c-*myc* and MMTV-*CDC37*+c-*myc* transgenic females.

To begin to address how *CDC37* and c-*myc* collaborate in transformation, we examined the levels of several protein kinases as well as c-*myc* in mammary carcinomas

from MMTV-*CDC37*+c-*myc* and MMTV-c-*myc* animals (Figure 6). As a control, we also examined the levels of proteins in MMTV-*ras* tumors. As expected, the level of Cdk4 was increased in tumors expressing MMTV-*CDC37*, relative to that found with MMTV-c-*myc* alone. Thus far, we have been unable to detect Raf-1 in tissues using the available antibodies but we did find that the levels of Erk1 were also increased. Unexpectedly, we found that c-*myc* levels were also increased in the presence of MMTV-*CDC37*, compared to animals expressing only MMTV-c-*myc*. The observed differences in protein levels cannot be explained by the increased number of dividing cells, since no significant difference was observed in the mitotic index of these tumors (data not shown). Tumors derived from MMTV-*ras* and MMTV-c-*myc* mice contained a single Cdc37 species while higher levels of a closely spaced doublet of Cdc37 protein was found in extracts from MMTV-*CDC37*+c-*myc* tumors (Figure 6). Multiple forms of Cdc37 have been observed proviously (Stepanova et al., 1996) and may reflect its phosphorylation (unpublished data, L.S. and J.W.H.).

CDC37 cooperates with _neu_ in increasing the mitotic index of tumors.

Neu is a potent oncogene and a large fraction of MMTV-*neu* mice display proliferative disorders in the mammary gland within 10 months of birth (Bouchard et al., 1989). We monitored MMTV-*neu* and MMTV-*CDC37*+*neu* mice for transformation. Since differences in transformation in breeding and virgin females has not been reported, we maintained a colony of virgin females. As seen in Figure 7A, the kinetics of tumor appearance was similar in MMTV-*neu* and MMTV-*CDC37*+*neu* females (T_{50}~350 days). All tumors were found to be mammary adenocarcinomas of either ductal or alveolar origin. Both single and double transgenic mice usually developed a single tumor mass

with no evidence of metastasis (Figure 7C). Histological analysis revealed that despite a similar gross appearance and incidence, MMTV-*neu* and MMTV-*CDC37+neu* tumors display dramatically different numbers of mitotic figures (Figure 7B). While mitotic figures were rare in MMTV-*neu* tumors (1-2 per microscopic field), we observed ~8 mitotic figures per field in tumors from MMTV-*CDC37+neu* mice (Figure 7D). These data indicate that CDC37 expression does not influence the frequency of transformation by neu but may enhance proliferative signals downstream of neu.

Inappropriate mammary duct development in male MMTV-*CDC37* mice.

Phenotypic analysis of mammary glands during development failed to identify significant differences between female MMTV-*CDC37* mice and their wild-type littermates, except for a 2-3 days delay in the rate of involution after lactation (data not shown). However, we did observe alterations in the development of male ductal systems, as assessed by whole-mount analysis. The degree of breast duct development varies in different mouse strains, ranging from the presence of the initial ductal sprout in some of the fat pads to relatively well-developed branching ductal tree. In our strain background, male mice do not develop a significant mammary duct structure, although the fat pad is well-developed. In contrast, 60-70% of the adult MMTV-*CDC37* male mice have well-formed breast ducts with different degrees of elaboration by the age of 7 months (Figure 8A, B). In the MMTV-*CDC37.2* line, which has lower levels of expression, 30-40% of male animals developed breast ducts in the inguinal fat pad by the age of 7 months. In control non-transgenic littermates of the similar mixed background, only 10% of adult males have non-branching initial sprout structure (Figure 8B).

To monitor the age-dependence of the effect, we performed whole-mount analysis of the male mammary glands at different ages (Figure 8B). This analysis demonstrated that 70% of 4 week old MMTV-*CDC37*.1 and control of animals have a tiny initial breast sprout which later would give raise to breast ducts. During the first 6 weeks after birth, this ductal sprout regressed in most of the non-transgenic animals, and the fraction that maintained a ductal sprout (10%) did not change up to 8 weeks (Figure 8B). In contrast, the percentage of MMTV-*CDC37* animals that maintain and elaborate ductal systems remain at ~70%. At 6 weeks of age, 70% of transgenic animals have about the same or somewhat better developed initial sprout, and by 8 weeks, 70% of transgenic animals have well-developed branching duct system, resembling the structures found in older males. There was no significant change in breast duct development between the ages of 8 weeks and 7 months in both transgenic and control groups.

Discussion

Proliferation requires the coordinate activation of multiple signaling pathways which ultimately converge on the cell cycle machinery to promote DNA replication and cell division. Studies in a variety of systems suggest that Cdc37 and Hsp90 are required to establish important signaling pathways through interaction with intrinsically unstable kinases, including the oncoprotein kinases Cdk4, Raf-1 and src family members (Stepanova et al., 1996; van der Straten et al., 1997; Grammatikakis et al., 1999; Xu et al., 1999). In this study, we have examined the proliferative role of *CDC37* through analysis of MMTV-*CDC37* transgenic mice. Remarkably, we found that *CDC37* alone could function to cause neoplastic transformation of both mammary epithelium and cells of the lymphoid compartment. In this context, *CDC37* functions as a weak oncogene with rates of transformation similar to that observed previously in MMTV-cyclin D1 mice (19-22 months onset) (Wang et al., 1994). Mammary tumors from these animals displayed low mitotic activity, consistent with their very slow development and growth. Two independent lines of MMTV-*CDC37* mice both displayed extensive transformation, but the tissue specificity of the MMTV-*CDC37*.2 line was broader than the *CDC37*.1 line (Table 1). Transgenic mice expressing cyclin E, cyclin D, and *ras* also display variability in the extent and tissue specificity of transformation (Bortner et al., 1997; Tremblay et al., 1989; Wang et al., 1994). This variability may reflect the site of integration and/or the levels of expression. We consider it likely that the persistent expression of *CDC37* may allow what would otherwise be silent somatic mutations occurring over time in these animals to give rise to transformation. *CDC37* appears to have multiple targets, many of which can promote proliferation in various settings. Thus, it is not clear whether the

multiple transformation events we have observed in MMTV-*CDC37* mice reflect mutational activation of a single collaborating pathway or mutations in different pathways in different tumors that occur stochastically.

c-*Myc* can collaborate with *ras* to transform a variety of cell types both *in vitro* and *in vivo* (Land et al., 1983; Sinn et al., 1987). The ability of *ras* to function as a growth promoter as opposed to a growth inhibitor may rely upon inactivation of the ARF-Mdm2-p53 pathway. In primary fibroblasts, *ras* can induce G1-arrest and a senescence-like state dependent upon p53 and $p16^{INK4a}$ but this activity is lost with immortalization (Serrano et al., 1997; Lin et al., 1998). The selective pressure on c-*myc* expressing cells to inactivate the ARF-Mdm2-p53 pathway or undergo apoptosis (Zindy et al., 1998), therefore, provides a plausible model for collaboration between *ras* and *myc* in cellular transformation (reviewed in Sherr, 1998). c-*Myc* may also promote proliferation by controlling Cdk activity. c-*Myc* expression can induce Cdk4/cyclin D kinase activity in certain situations (Mateyak et al., 1999) and there is evidence that Cdk4 is required for the proliferative effects of c-*myc* in some systems (Haas et al., 1997). In addition, c-*myc* expression leads to cyclin E/Cdk2 kinase activation, at least in part through inactivation of p27 (Vlach et al., 1996; Leone et al., 1997; Perez-Roger et al., 1997). Because of the link between Raf-1, Cdk4 and Cdc37, we asked whether *CDC37* could cooperate with c-*myc*-dependent transformation by breeding MMTV-*CDC37* and MMTV-*c-myc* mice. In principle, stabilization and/or activation of Raf-1 by ectopic Cdc37, which has been observed in heterologous systems (Grammatikakis et al., 1999), could inappropriately activate the ras pathway and this could be observed as collaboration with c-myc in vivo. We found that *CDC37* and c-*myc* collaborate to transform multiple tissues in both

breeding and non-breeding females, as well as in males, and both MMTV-*CDC37* lines behaved similarly in this regard. In females, *CDC37* enhanced both the rate and extent of mammary transformation by c-*myc*, independent of hormonal effects due to pregnancy. Importantly, the number of tumor foci observed with c-*myc* in the presence of MMTV-*CDC37* was dramatically increased (from an average of 3 tumors/animal to an average approaching 20/animal). This result suggests that in some cell types, *CDC37* expression may be rate limiting for transformation. In this regard, we have observed expression of *CDC37* in c-*myc* and *ras* induced mammary tumors, despite the fact that *CDC37* is not expressed in resting mammary epithelium. The increased rates of mammary transformation observed with pregnancy with MMTV-c-*myc* and MMTV-*ras* may reflect the fact that *CDC37* is induced during pregnancy (Stepanova et al., 1996) and could provide a proliferation-permissive setting that allows for mutations that promote transformation. We expect that other events including inactivation of the ARF/p53 pathway (reviewed in Sherr, 1998) are also involved in c-*myc* mediated transformation in MMTV-*CDC37* mice.

An interesting aspect of this study was the finding that Cdc37 expression allowed transformation by c-*myc* in cell types where it is normally not oncogenic. In virgin females, MMTV-*CDC37*+c-*myc* mice developed salivary tumors. Although MMTV-*ras* mice develop salivary tumors (Mangues et al., 1990), MMTV-c-*myc* mice have not been reported to develop salivary tumors. The inability of c-*myc* to transform the salivary epithelium is considered a peculiarity of this oncogene. Our results suggest that the absence of *CDC37* expression in adult salivary glands may contribute to the inability of c-myc to transform this tissue. In addition, we found that expression of *CDC37* allows c-

myc to transform Leydig cells in the testis. c-*Myc* induced a very mild hyperplasia in a small fraction of animals but when *CDC37* was co-expressed, there was a dramatic increase in the extent and severity of Leydig cell hyperplasia. Moreover, 30% of the animals examined displayed evidence of overt neoplasia in the testis. In contrast with c-*myc*, MMTV-*CDC37* did not affect the rate of mammary transformation induced by MMTV-*neu*. This likely reflects difference in mechanism between these two oncogenes and suggests the possibility that c-neu may be capable of activating *CDC37* expression. Although there was no effect on tumor incidence, we did find an increase in mitotic activity in MMTV-*CDC37+neu* mammary tumors relative to MMTV-*neu* tumors. The biochemical basis for this is not clear at present.

In addition to the oncogenic effects of *CDC37* expression, we found that a significant proportion of adult transgenic males develop an extensive system of mammary ducts resembling that of a virgin female mouse. The development of rudimentary mammary ducts begins during embryonic development. Sexual dimorphism is already pronounced at embryonic day 14 when the male anlage undergoes significant cell death caused by androgens (Kratochwil, 1975; Sakakura, 1987). The degree of destruction of the initial sprout varies greatly among strains. In our outbred strain (B6D2F1 x ICR), the initial sprout could be detected in inguinal mammary fat pads of about 70% of all newborn males. This number dropped sharply at the age of 4 weeks in non-transgenics and later could be detected in inguinal pads of only 10% of the adult animals, in which it remained without any significant additional growth. In transgenic males, however, the initial sprout observed in 4 week old transgenic males did not undergo normally programmed necrosis/apoptosis, and instead, it continued to

proliferate, ultimately forming an immature ductal system containing a few side-branches. The mechanism underlying this developmental alteration is not known at present but could reflect effects of *CDC37* on the androgen receptor, as has been observed in budding yeast (Fliss et al., 1997).

Although the phenotypic consequences of *CDC37* expression and collaboration with c-*myc* are striking, the biochemical mechanisms underlying its action are likely to be complex, possibly involving multiple kinase pathways that function interdependently to promote proliferation. Stabilization and/or activation of Cdk4 or Raf, the two most prominent targets of Cdc37, could result in both activation of the ras pathway and activation of Cdks. In the later case, increased Cdk4 levels could simultaneously sequester $p16^{INK4a}$ and promote proliferation via activation by cyclin D. This could, in turn, lead to activation of cyclin E/Cdk2 by both increasing cyclin E expression and by sequestration of p27. We have observed increased levels of both Cdk4 and the Erk1 kinase, but have not been able to assess the levels of Raf due to lack of suitable antibodies. Interestingly, we noticed that mammary tumors from MMTV-*CDC37*+c-*myc* animals contained significantly higher levels of c-*myc* than tumors from MMTV-c-*myc* animals without changes in mitotic index. Recent studies suggested that activation of the *ras* pathway stabilizes c-*myc* (Sears et al., 1999). It is therefore possible that *CDC37*, through its interaction with kinases in the *ras* pathway, indirectly stabilizes c-*myc*. Further studies are required to determine whether increased levels of c-*myc* via *CDC37* expression are an important component of the collaborative effects seen in vivo. We also note that the role of *CDC37* in transformation suggested by this work may explain the sensitivity of various tumor types to anzamycin antibiotics such as geldanamycin and

herbimycin (Murakami et al., 1988; Whitesell et al., 1992, 1994; Auvinen et al., 1995; Kwon et al., 1995), which are known to bind Hsp90 and disrupt *ras* and cyclin D dependent signaling pathways.

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Materials and Methods

Generation of transgenic mice.

An MMTV-*CDC37* transgene was generated by cloning a Xho I fragment containing 1.6-kb *CDC37* open reading frame (ORF) into a plasmid containing an MMTV promoter, beta-globin splice sequences and bGH polyadenylation sequences. The 4.63-kb transgene fragment was released from vector by digesting with Not I/Kpn I and purified. Transgene DNA was microinjected into male pronuclei of B6D2F1 mouse embryos in the Baylor College of Medicine transgenic core facility. Resulting pups were screened by Southern analysis of genomic DNA isolated from mouse tails digested with BamHI. To establish lines of transgenic mice, founders were continuously mated with ICR mice. Non-transgenic littermates of heterozygous parents were used as controls.

Northern analysis.

Total RNA was prepared from mouse tissues, separated on 1% agarose gel, transferred to Hybond N+ (Amersham) membrane, and blotted with a ^{32}P-labelled *CDC37* cDNA probe to detect endogenous and transgene derived transcripts or a 5'+3' probe consisting of rabbit beta-globin splice site sequences and bovine polyadenylation signal DNA, which was used to detect only exogenous *CDC37* transcripts. Blots were stripped and reprobed with a GAPDH probe to control for RNA levels.

Generation of double transgenic mice.

MMTV-*CDC37* heterozygous females were mated with MMTV-c-*myc* (Charles River Laboratory) or MMTV-*neu* (Jackson Labs) homozygous transgenic males. Both MMTV-c-*myc* and MMTV-*neu* mice were on a inbred FVB genetic background. Resulting progeny carried either both transgenes (c-*myc*+*CDC37* or *neu*+*CDC37*) or a single

transgene (c-*myc* or *neu*). To control for variability of the mixed genetic background in the resulting progeny, littermates with single transgenes were monitored for comparison in all experiments. For non-transgenic controls, MMTV-*CDC37* heterozygous females were crossed with non-transgenic FVB males.

Histology and immunohistochemistry.

For histological analysis, mouse tissues were excised, fixed in 4% formaldehyde in PBS overnight at +4^{0}C, prior to being embedded in paraffin. Embedded tissues were sectioned at a thickness of 5μm, and stained with hematoxylin and eosin (H&E). For immunohistochemistry, 5 μm sections were stained with rabbit polyclonal affinity purified Cdc37 antibodies as described previously (Stepanova et al., 1996).

Western blot analysis.

Frozen tumor specimens were used for preparation of protein lysates by homogenization in NP-40 buffer (Stepanova et al., 1996) followed by centrifugation and determination of protein concentration by Bradford assays. For western blotting, 200 μg of extract was run through 12.5% SDS-PAGE and transferred to nitrocellulose. Blotting was performed using polyclonal Cdc37 antibodies (Stepanova et al., 1996) or Cdk4, Erk1/2, and c-myc antibodies from Santa Cruz. Detection was accomplished using HRP-conjugated secondary antibodies in combination with ECL (Amersham).

Whole-mount analysis.

Inguinal fat pads were excised from the animals, spread on a glass surface and fixed in 10% Formalin for 10-12 hours, washed in acetone for 48 hours, followed by wash in 100% and 95% EtOH for 1 hour each. Tissues were stained for 12 hours in a solution containg 4.9 ml hematoxylin (5g Hematoxylin in 50 ml of 95% EtOH); 0.56 gm FeCl in

150 ml of distilled H20; 100 ml of 1N HCl and 1350 ml of 95% EtOH. Stained tissues were washed for 1 hour in distilled water and increasing concentrations of EtOH (70-100%), and finally in Xylene. Tissues were stored in glass vials, covered with Methyl Salicylate.

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FIGURE LEGENDS

Figure 1. Characterization of MMTV-*CDC37* transgene expression.

(A) Structure of the construct used to generate MMTV-*CDC37* mice (see Materials and Methods for details).

(B) Southern blot analysis of MMTV-*CDC37*.1 and MMTV-*CDC37*.2 transgenic lines. Tail DNA was digested with *Bam HI* prior to Southern analysis with the *CDC37* cDNA. The 1.6 kb band corresponds to the construct fragment containing rabbit β-globin splice site and 0.9 kb bands represents fragment containing bovine polyadenylation signal [see (A)]. We estimate 15 and 8 copies of the transgene for MMTV-*CDC37*.1 and *CDC37*.2, respectively.

(C) Northern blot analysis of *CDC37* expression in different tissues derived from transgenic and control animals. Total RNA was hybridized with both the *CDC37* cDNA which detects both endogenous *CDC37* and the transgene derived message and a probe containing 5' and 3' sequences (5' + 3') which specifically detect transgene expression. The GAP-DH probe (glyceraldehyde 3-phosphate dehydrogenase ORF) is used as a loading control. Muscle tissue has intrinsically higher levels of GAP-DH mRNA.

Figure 2. Histology of the tumors developed by MMTV-*CDC37* transgenic mice.

(A) Ductal adenocarcinoma of the mammary gland with arrows indicating squamous differentiation. For (A), (B) and (C): a, 100 x magnification, hematoxylin and eosin (H&E) staining; b, 400 x magnification, H&E; c, 1000 x field stained with anti-Cdc37 antibodies and visualization with FITC conjugated secondary antibodies; and d, 1000 x, DAPI staining of the same field as (c).

(B) Ductal adenocarcinoma of the mammary gland. Arrows indicate protein secretion.

(C) Lymphoma in the mammary gland. Small arrows point to the capsule encircling a lymph node. Note the lymphocytes invading the surrounding stroma of the mammary gland. Large arrows indicate normal mammary ducts.

Figure 3. Cooperation between *CDC37* and c-*myc* oncogenes in female mice.

(A) Quantitation of incidence of proliferative disorders. Tumor-free animals from breeding females are shown in black while tumor incidence in virgin animals is shown in red. T_{50} corresponds to the time (days) at which 50% of all females in the group develop detectable tumors, 'n' indicates the number of animals in each group. Cdc37 mice used in this experiment were from the MMTV-*CDC37*.1 line. Breeding and virgin MMTV-*CDC37*/c-*myc* females bore either MMTV-*CDC37*.1 or MMTV-*CDC37*.2 transgene.

(B) Types of tumors developed by MMTV-*CDC37*, MMTV-c-*myc*, and double transgenic MMTV-*CDC37*+c-*myc* mice. The percentage of the animals developing each type of tumor from (A) is shown. Some of the animals developed more than one type of malignancy.

(C) Gross appearance of the breeding females expressing either MMTV-c-*myc* (right) or MMTV-*CDC37*+c-*myc*. The double transgenic females develop more tumors per animal than do single c-myc transgenics. The additional tumors, which we not visible by gross examination, were detected by detailed histopathological analysis.

(D) Quantitation of tumor number/animal. The percentage of animals developing a given number of mammary adenocarcinomas is shown. MMTV-*CDC37* animals developed only one tumor per animal. c-*Myc* expressing animals developed from one to four, while

the majority of double transgenics had more than nine tumors/animal. The number of tumors was estimated by counting foci on the sections from fixed preparations of all mammary glands.

(E) Histology of a mammary adenocarcinoma developed by MMTV-c-*myc* breeding female. For (E) and (F): a, 100 x magnification, hematoxylin and eosin (H&E) staining; b, 400 x magnification, H&E; c, 1000 x field stained with anti-Cdc37 antibodies and visualization with FITC conjugated secondary antibodies; and d, 1000 x, DAPI staining of the same field as (c).

(F) Histology of a mammary adenocarcinoma developed by MMTV-*CDC37*+c-*myc* breeding female. The level of *CDC37* expression in the tumor developed by a double transgene female is high and has uneven distribution throughout the tumor, while every cell in the MMTV-c-*myc* derived tumor expresses the same medium level of *CDC37*.

Figure 4. *CDC37* cooperates with c-*myc* in induction of salivary gland adenocarcinomas in virgin females.

(A) *CDC37* expression is absent in the salivary gland of a non-transgenic female. For (A), (B), and (C): a, 400 x magnification, H&E; b, 1000 x field, staining with rabbit anti-Cdc37 polyclonal antibodies and visualization with anti-rabbit antibodies conjugated with FITC fluorescence marker; c, 1000 x, nuclear DAPI staining of the same field as (c).

(B) Expression of MMTV-driven *CDC37* in the morphologically normal salivary gland of a transgenic female mouse.

(C) Salivary gland adenocarcinoma in a double transgenic MMTV-*CDC37*+c-*myc* virgin female. The tumor has a large number of mitotic figures indicating high mitotic activity.

Figure 5. *CDC37* cooperates with c-*myc* in induction of the Leydig cell hyperplasia and transformation.

(A) MMTV-*CDC37*+c-*myc* double transgenic males develop tumors, while male animals expressing a single transgene are unaffected. Plot of tumor-free mice over time. Three of 4 tumors were Leydig cell tumors while the fourth was a lymphoma.

(B) MMTV-*CDC37*+c-*myc* double transgenic males display extensive Leydig cell hyperplasia compared to MMTV-c-*myc* and MMTV-*CDC37* animals. Histological sections of testis derived from grossly unaffected males were analyzed at 400 days of age. Number of animals in each group is shown.

(C) Tissue section of a normal testis with arrows indicating the position of Leydig cells located between seminiferous tubules with active spermatogenesis: a) 100 x magnification, H&E staining; b) 400 x magnification, H&E staining to show the usual number and morphology of Leydig cells.

(D) Expression of *CDC37* in the testis of MMTV-*CDC37* transgenic (a,b) or non-transgenic (c,d) male mice at 1000 x magnification: (a, c) *CDC37* expression in the cytoplasm of Leydig cells; (b, d) DAPI staining to identify nuclei.

(E) Mild hyperplasia found in 20% of 400 day old males expressing MMTV-c-*myc*.

(F) High grade hyperplasia found in 75% of 400 day old MMTV-*CDC37*+c-*myc* mice.

(G) Example of a Leydig cell tumor found in MMTV-*CDC37*+c-*myc* mice: a) 100 x magnification, H&E; b) 400 x magnification, H&E; c) 1000 x field stained with anti-Cdc37 antibodies; and d) 1000 x, DAPI staining of the same field as (c) to identify nuclei.

Figure 6. MMTV-*CDC37*+c-*myc* mammary tumors have higher levels of c-*myc* and multiple signaling proteins than tumors from MMTV-c-*myc* animals. Protein extracts (250 μg/lane) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the indicated antibodies.

Figure 7. Co-expression of *CDC37* enhances the mitotic activity of tumors induced by MMTV-*neu*.

(A) MMTV-*CDC37* does not increase tumor incidence in virgin female MMTV-*neu* transgenic mice. All tumors developed by single and double transgenics were mammary adenocarcinomas of ductal and alveolar origin. The graph shows the number of tumor-free animals as a function of age.

(B) The mitotic index is significantly increased in the mammary tumors derived from MMTV-*neu* tumors co-expressing MMTV-*CDC37*. Mitotic figures are indicated by arrows on the 400 x field of H&E stained tumor sections.

(C) The number of tumors per animal is not significantly altered in MMTV-*CDC37*+*neu* animals when compared with MMTV-*neu* animals. The percentage of animals developing a given number of tumors/animal is calculated, and the number of animals in each group is shown.

(D) Quantitation of mitotic indices. Tumors from MMTV-*CDC37*+*neu* mice have more mitotic figures per 400x microscopic field. The averages of 20 fields are shown with maximal and minimal values indicated for each genotype.

Figure 8. Inappropriate mammary duct development in MMTV-*CDC37* transgenic males.

(A) Whole-mount analysis of the mammary glands from transgenic males and non-transgenic littermates at 7 months of age. Inguinal mammary glands were fixed in formalin, cleared with acetone, and stained with hematoxylin to visualize mammary ducts. By 7 months, a significant number of transgenic males developed an extensive system of breast ducts resembling that of a normal virgin female, while only 10% of the males in the control group had retained an initial sprout. LN - lymph node. Black arrows - mammary ducts in transgenic males. Clear arrow - initial duct sprout in a non-transgenic male.

(B) The percentage of transgenic and non-transgenic animals retaining breast structures as a function of age. For each time point, more than 10 inguinal mammary glands were autopsied and analysed.

Table 1. Neoplasms found in transgenic females carrying the MMTV-*CDC37*

Pathology (no./total)	Line	% of mice affected
Mammary ductal adenosquamous carcinoma	MMTV-*CDC37.1*	60 (6/10)
	MMTV-*CDC37.2*	9 (1/11)
Mammary ductal adenocarcinoma	MMTV-*CDC37.1*	10 (1/10)
	MMTV-*CDC37.2*	0 (0/11)
Lung adenocarcinoma	MMTV-*CDC37.1*	0 (0/10)
	MMTV-*CDC37.2*	9 (1/11)
Lymphoma	MMTV-*CDC37.1*	50 (5/10)
	MMTV-*CDC37.2*	18 (2/11)
Liposarcoma	MMTV-*CDC37.1*	0 (0/10)
	MMTV-*CDC37.2*	19 (2/11)
Sarcoma	MMTV-*CDC37.1*	0 (0/10)
	MMTV-*CDC37.2*	10 (1/10)
Total affected	MMTV-*CDC37.1*	100 (10/10)
	MMTV-*CDC37.2*	63 (7/11)

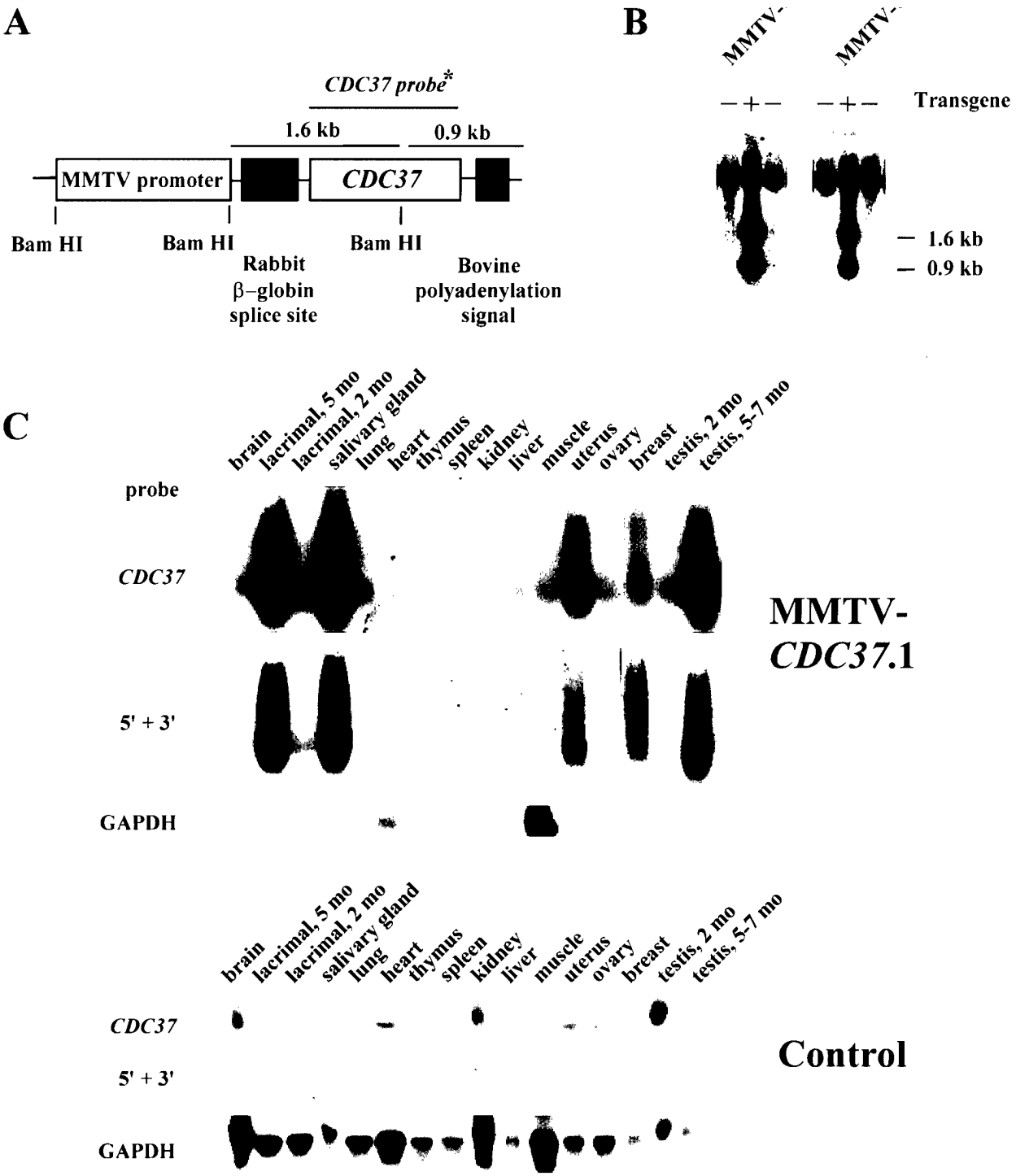

A
CDC37 probe*
1.6 kb
0.9 kb
MMTV promoter
CDC37
Bam HI
Bam HI
Bam HI
Rabbit β–globin splice site
Bovine polyadenylation signal
B
MMTV-
MMTV-
−+−
−+−
Transgene
1.6 kb
0.9 kb
C
brain
lacrimal, 5 mo
lacrimal, 2 mo
salivary gland
lung
heart
thymus
spleen
kidney
liver
muscle
uterus
ovary
breast
testis, 2 mo
testis, 5-7 mo
probe
CDC37
5' + 3'
GAPDH
MMTV-CDC37.1
brain
lacrimal, 5 mo
lacrimal, 2 mo
salivary gland
lung
heart
thymus
spleen
kidney
liver
muscle
uterus
ovary
breast
testis, 2 mo
testis, 5-7 mo
CDC37
5' + 3'
GAPDH
Control

Figure 1

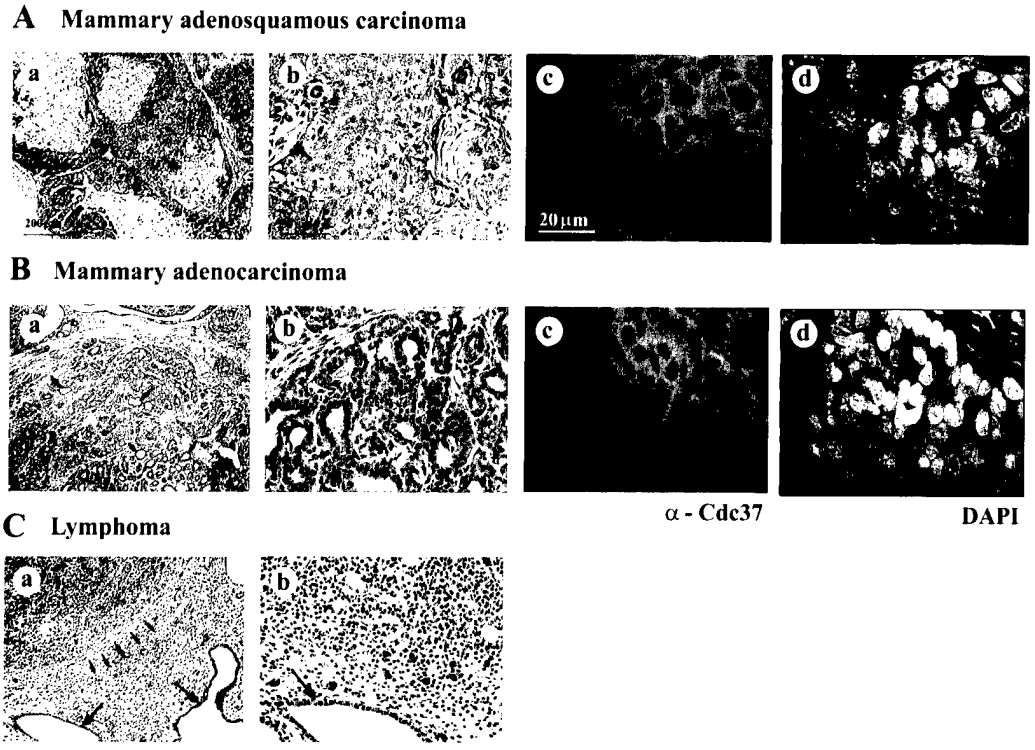


Figure 2
(Stepanova et al., 1999)

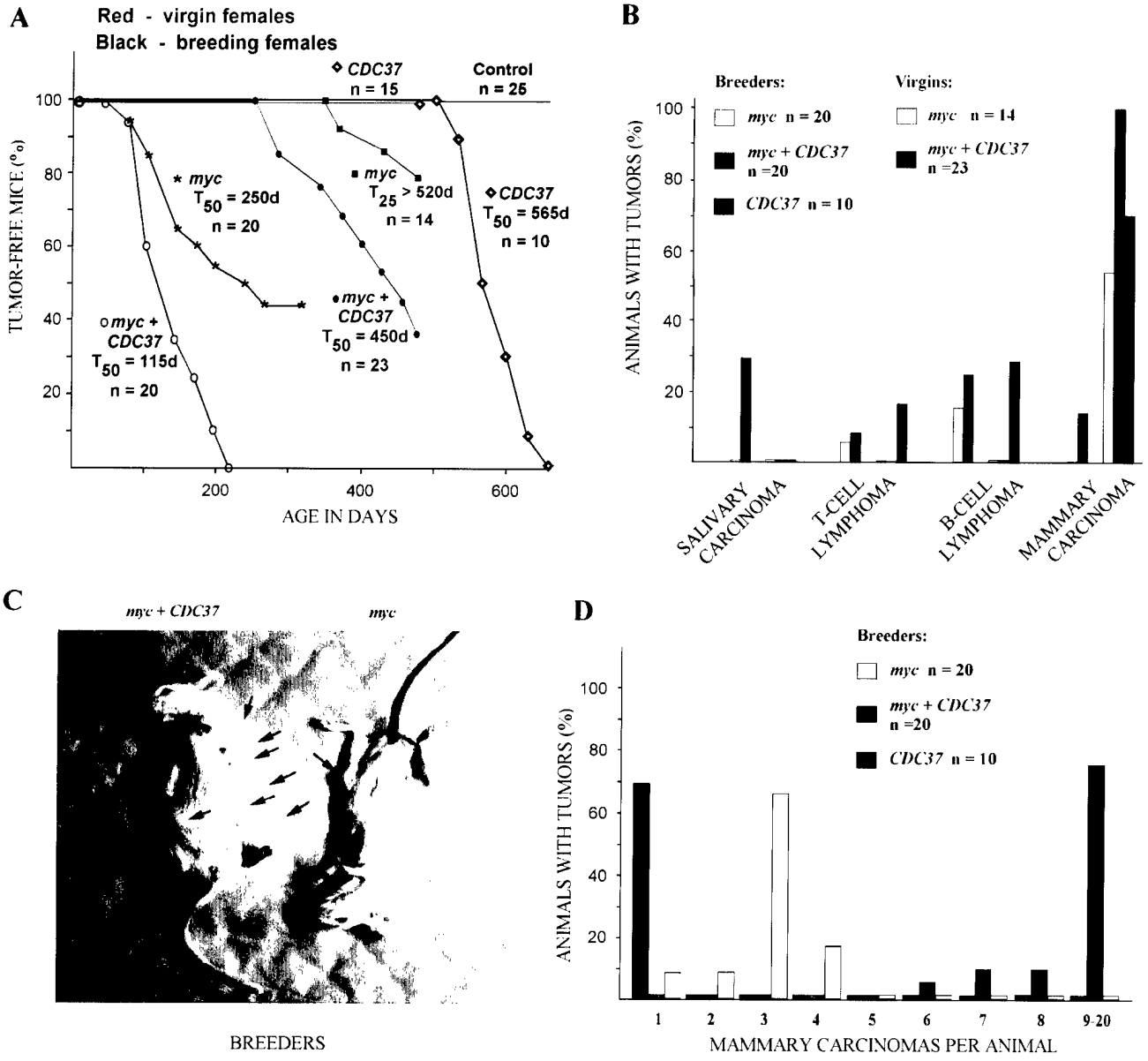


Figure 3 (1)
(Stepanova et al., 1999)

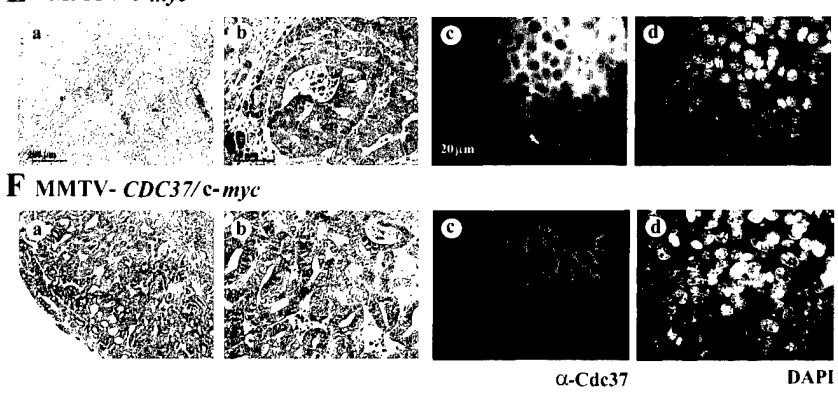


Figure 3(2)
(Stepanova et al., 1999)

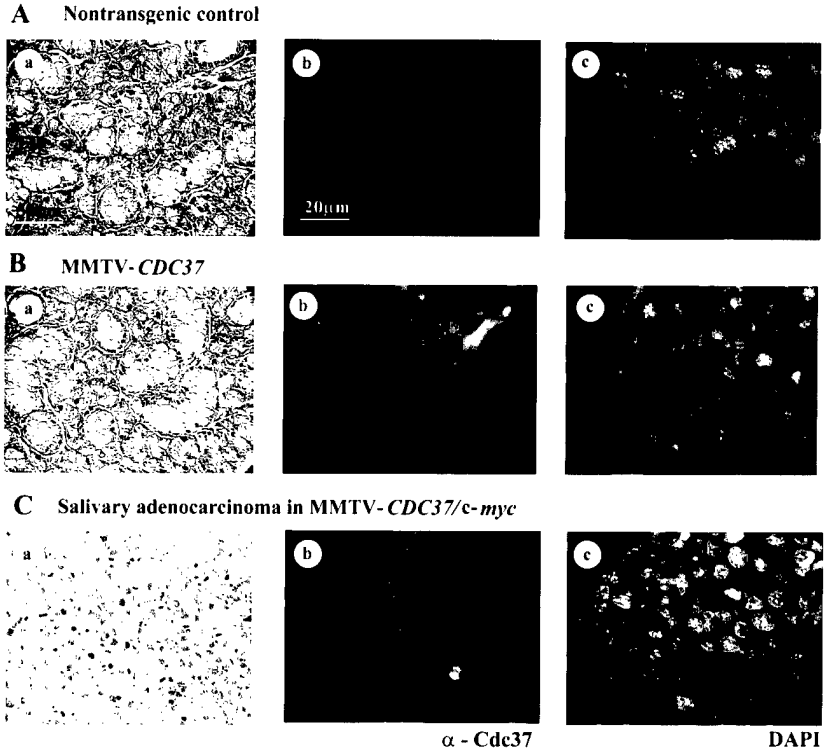


Figure 4
(Stepanova et al., 1999)

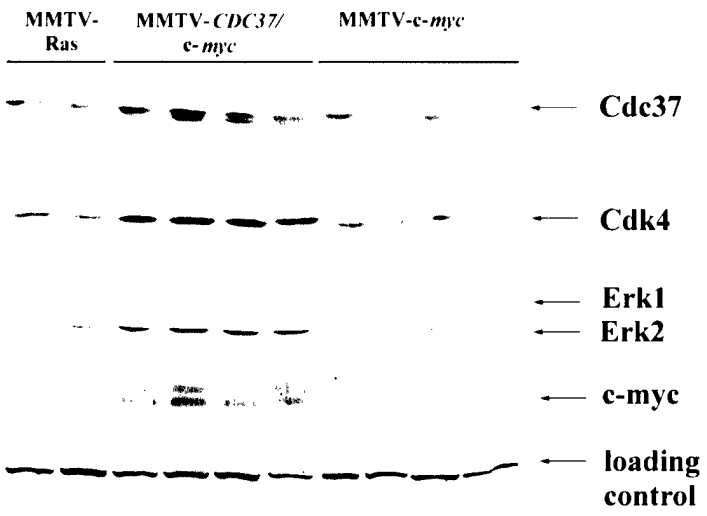
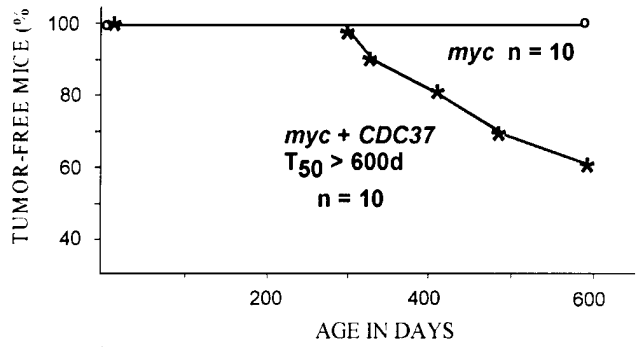


Figure 5
(Stepanova et al., 1999)


TUMOR-FREE MICE (%
100
80
60
40
200
400
600
AGE IN DAYS
myc n = 10
myc + CDC37
T50 > 600d
n = 10

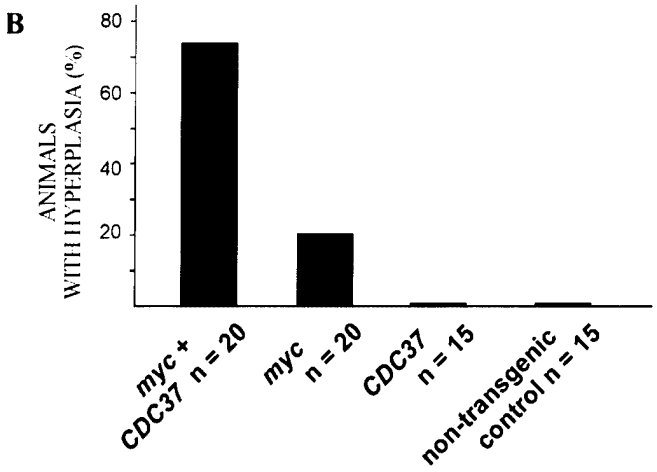

B
ANIMALS WITH HYPERPLASIA (%)
80
60
40
20
myc + CDC37 n = 20
myc n = 20
CDC37 n = 15
non-transgenic control n = 15

Figure 6(1)

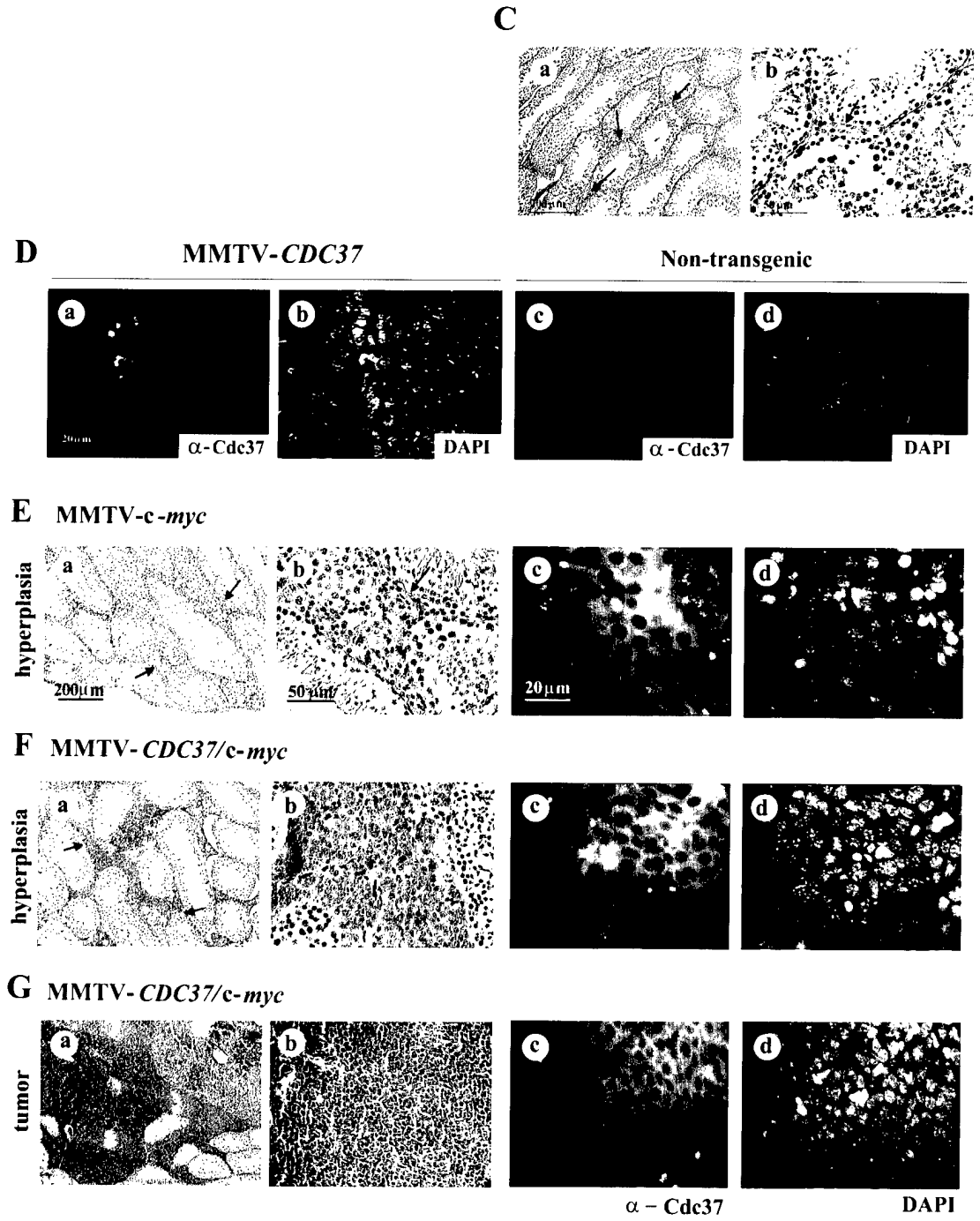


Figure 6(2)
(Stepanova et al., 1999)

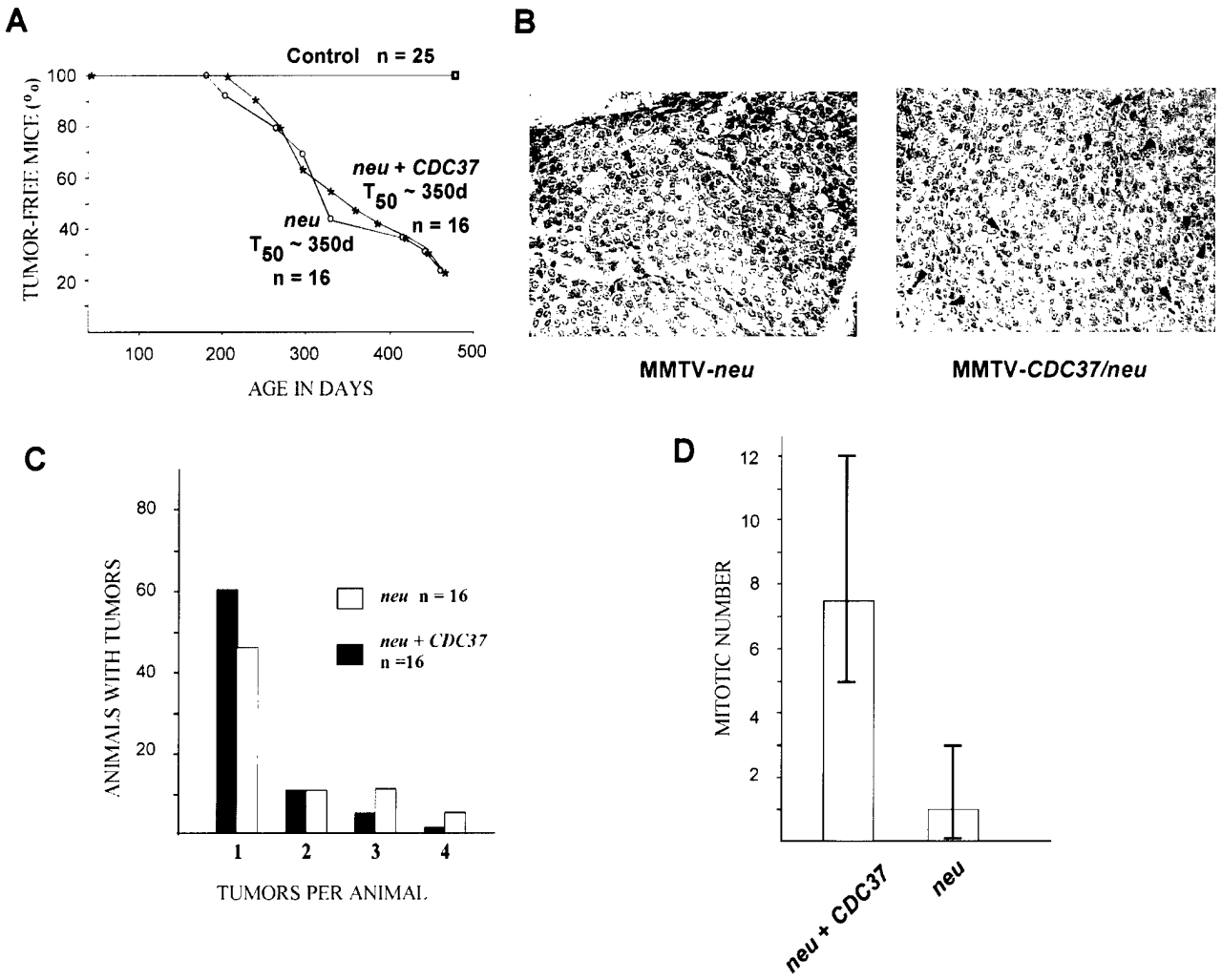


Figure 7
(Stepanova et al., 1999)

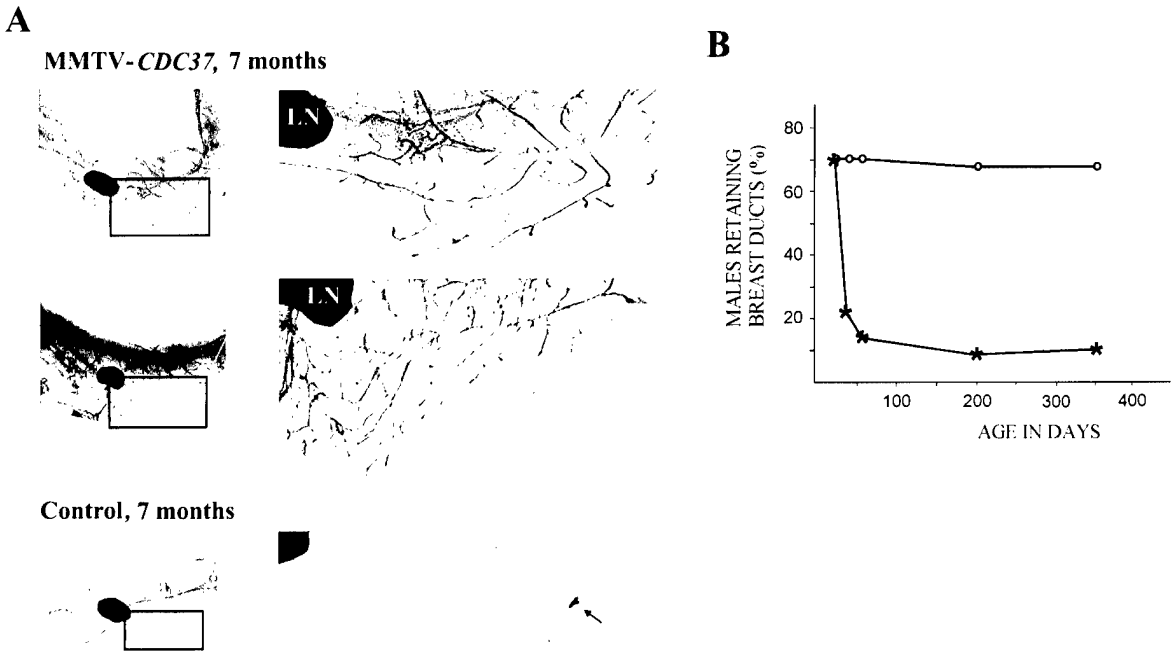


Figure 8
(Stepanova et al., 1999)